

Capsular Polymer of *Haemophilus influenzae*, Type b

I. STRUCTURAL CHARACTERIZATION OF THE CAPSULAR POLYMER OF STRAIN EAGAN

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SUMMARY

The capsular type-specific antigen of *Haemophilus influenzae*, type b, has been reported to have a unit structure composed of D-ribose and phosphate. Recently, the presence of ribitol was found in preparations of type b capsular antigen. Our analytical results show equimolar proportions of ribose, ribitol, and phosphate. Periodate oxidation studies, paper chromatography of acidic and alkaline hydrolysates, and NMR spectral data indicate the structure of the capsular antigen of *H. influenzae* b, strain Eagan to be a polyribosyl-ribitol phosphate polymer.

MATERIALS AND METHODS

Biochemicals—Ribitol was obtained from Nutritional Biochemicals Corp., Cleveland, O. Ribose 5-phosphate, monosodium salt dihydrate, was purchased from Calbiochem, Los Angeles, Calif., and D-ribose from Applied Science Laboratories, Inc., State College, Pa. Methyl- α -D-glucoside was purchased from Matheson, Coleman and Bell, Cincinnati, O. Ribitol 5-phosphate was prepared by hydrogenation of ribose 5-phosphate (12) and 1,4-anhydribitol was prepared from ribitol (13). Ribose 2-phosphate and ribose 3-phosphate were prepared by hydrolysis of adenosine 2'-monophosphate or adenosine 3'-monophosphate obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis. Hydrolysis was done with Dowex 50 (14). HI-B was isolated according to the method of Anderson *et al.* (6) from the Eagan strain of *Haemophilus influenzae* type b; also supplied to us by Anderson. Molecular size and homogeneity were estimated by means of Sepharose 4B gel filtration on columns 2.5 cm in diameter and 30 cm in length (Pharmacia Fine Chemicals, Piscataway, N.J.). Sedimentation velocity values were done, using a 4.544 mg/ml solution of HI-B in 0.1 M sodium phosphate buffer, pH 6.8. Runs were done at 20°, at 60,000 rpm in the model E Beckman analytical ultracentrifuge using schlieren optics.

Acid and Base Hydrolysis—Acid hydrolysis was done on samples of 5 to 10 mg at a concentration of 5.0 mg/ml under various conditions as follows: 0.1 M HCl at 100° for 10 min, 1.0 M HCl at 100° for 20 min, or 2.0 M HCl at 100° for 17 or 66 hours. Excess HCl was removed by repeated evaporation under vacuum on a rotary evaporator until pH 3.0 was reached. Base hydrolysis was performed on 5- to 10-mg samples at a concentration of 5.0 mg/ml with 0.5 M NaOH for 4 hours at 25°. Sodium ions were removed by passage through Dowex 50W-X2 resin, 50 to 200 mesh, in NH_4^+ form (15).

Paper Chromatography—Whatman No. 1 paper, washed in 2 M acetic acid, and then water, was used for descending paper chromatography. The solvent systems and times of development used were: Solvent A, propanol/ammonia (specific gravity 0.88)/water, 6/3/1, 24 hours; Solvent B, 1-butanol/pyridine/water, 6/4/3, 24 hours; Solvent C, 1-butanol/acetic acid/water, 3/1/1, 24 hours; Solvent D, 1-butanol/ethanol/water/ammonia (specific gravity 0.88), 40/10/49/1, 18 hours. Reducing sugars were detected on paper chromatograms by spraying with aniline-oxalate reagent (16). Phosphoric acid esters were detected by molybdate-acetone staining (17). Glycols were detected by periodate-Schiff reagent (18).

Ion Exchange Chromatography—To affect the separation and identification of ribose 2- and 3-phosphates and other hydrolysis products, acid and basic hydrolysates of 8 to 10 mg of HI-B were chromatographed on an ion exchange column (approximately 0.5 cm \times 10 cm) of Dowex 1-C1 (14). The samples were at pH 8.0 when applied to the column. Compounds not containing phosphate esters did not absorb and were removed with water. Elution and separation of ribose 2- and 3-phosphates was achieved with 1 liter of a buffer consisting of 0.04 M NH_4Cl and 0.004 M $\text{K}_2\text{B}_4\text{O}_7$; other products were eluted in a buffer of 0.06 M NH_4Cl and 0.004 M $\text{K}_2\text{B}_4\text{O}_7$. Flow rates were about 2.5 ml/min. To detect both free

In 1953, Zamenhof *et al.* (1) postulated that the type-specific substance of *Haemophilus influenzae*, type b, was composed of polyribosephosphate chains with 3:5-phosphate diester linkages between the ribose moieties. Two of these chains were linked together by 1:1'-glycosidic bonds. Further publications (2-5) established the double ribose unit as β -D-Ribf- β -D-Ribf. Anderson *et al.* (6) and Schneerson *et al.* (7) have also reported equimolar ratios of phosphorus and ribose, but Anderson could only account for two-thirds of the total weight. Schneerson *et al.* (7) reported that *Escherichia coli* antigens from certain strains were cross-reactive with *H. influenzae* type b capsular polymer (HI-B). The *E. coli* polymers precipitated 40% of the anti-type b antibodies in anti-*H. influenzae* type b sera. Two of the *E. coli* strains that cross-reacted with the HI-B had polymers containing ribose and ribitol. Chromatographic analysis of HI-B by these workers (8) yielded a spot with a mobility similar to ribitol. Argaman *et al.* (9-11) also found cross-reactivity of HI-B with ribitol-containing polymers of gram-positive bacteria, especially *Bacillus subtilis*, *Bacillus pumilus*, and *Staphylococcus aureus*. He suggested that his serologic findings indicated the presence of a polyribitolphosphate polymer separate from the polyribosephosphate polymer. This paper is concerned with the confirmation of the presence of ribitol in HI-B, its quantitative determination, and a proposed structure for HI-B isolated from *H. influenzae* b, strain Eagan based on periodate oxidation studies, paper chromatography, and ^{13}C NMR spectral analysis.

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TABLE I
Analyses of HI-B

Lot No.	S ^a values	K _{av} values	Molecular weight ^b	Sodium	Phosphorus	Ribose	Ribitol	Weight % of total
C10-D25-140	1.50	0.356	570,000	NT	2.58	2.48	2.32	90.3
C10-D25-154		0.364	520,000	2.44	2.46	2.59	2.50	93.0
C10-D25-179	0.88	0.770	21,500	NT	2.54	2.46	2.36	90.1

^a S values obtained from Beckman model E analytical ultracentrifuge data.

^b Based on K_{av} values obtained from Sepharose 4B gel filtration.

ribose and ribose-containing compounds, samples were assayed by the orcinol procedure.

Analytical Methods—Ribose and ribose phosphates were determined by a modification of the Bial's orcinol test (19). Ninhydrin was used to assay for the presence of nitrogen (20). Phosphorus was determined with a micromethod using molybdate (21). The Perkin-Elmer model 303 atomic absorption spectrophotometer was used to assay for sodium according to instrument specifications, with 36 nm lithium as the background. Ribitol was determined quantitatively using an assay for glycerol by Hanahan and Olley (22) with the following modification. The sample was treated with 0.1 M HCl for 20 min at 100°, before the timed reaction with sodium periodate. Periodate consumption was measured by the method of Fleury and Lange (23) on 100-mg samples dissolved in 100 ml of 0.1 M sodium phosphate buffer, pH 6.5. Formaldehyde production during periodate oxidation was measured by the chromotropic acid method (24). Formic acid production was measured by the method of Rolski and Maciak (25). Nucleic acids were estimated by the adsorption of a 1.0 mg/ml solution of HI-B at 260 nm. The absorbance of 50 µg of nucleic acid in 1 ml of water in a cell of 1-cm light path was assumed to be equal to 1.0. Quantitative precipitin curves were done according to the procedure of Schiffman (20). Antiserum to *H. influenzae* b was obtained from the Hyland Division of Travenol Laboratories, Costa Mesa, Calif.

NMR Spectral Analysis—¹³C NMR spectra were obtained on a JEOL-PS-100 spectrometer operating in Fourier transform mode at a frequency of 25.1405 MHz. Pulse width was 11 µs (45°) with a probe temperature of 45°. Computer data were accumulated using a 2500-Hz sweep width in 8192 data points and a recycle time of 2.0 s. Chemical shifts were measured relative to 2% internal dioxane and converted to the tetramethylsilane scale using $\delta_{\text{dioxane}} = +67.4$ ppm. The spectra were run on 2 ml of a solution containing 140 mg of HI-B in 1.5 ml of D₂O.

RESULTS

All studies on HI-B were done on three preparations which contained less than 0.5% nitrogen and less than 0.5% nucleic acid. These preparations had molecular weights of 21,000, 520,000, and 570,000, and K_{av} values of 0.770, 0.364, and 0.356, respectively (Table I), when compared to dextran 2000 on Sepharose 4B columns. The first and third preparations were homogeneous in size by this gel filtration technique. The second preparation had a small leading peak of larger molecular size, which may account for the difference observed with precipitin curves with specific antisera (Fig. 1). Ultracentrifuge studies on the first and third lots had S values of 1.5 and 0.88, respectively, under the conditions stated in "Materials and Methods." The two preparations of 520,000 and 570,000 molecular weight reacted with *Haemophilus influenzae* b antiserum to give a precipitin reaction; whereas, the 21,000 molecular weight preparation reacted only feebly, if at all (Fig. 1).

Quantitative determinations for sodium, phosphorus, ribose, and ribitol showed that these constituents were present in equal molar ratios (Table I). In the analysis of the three different HI-B preparations mentioned above, 90.1 to 93.0% of the total weight was accounted for. Samples weighing 8 to 10 mg each were dissolved in water to make 1.0 mg/ml solutions. Aliquots were

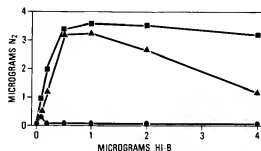


Fig. 1. Quantitative precipitin curves for Lot C10-D25-154, molecular weight 520,000 (■—■); Lot C10-D25-140, molecular weight 570,000 (▲—▲); and Lot C10-D25-179, molecular weight 21,500 (●—●) with antiserum to *Haemophilus influenzae* b.

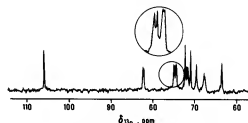


Fig. 2. ¹³C NMR spectrum of HI-B, 140 mg in 1.5 ml, in D₂O. The spectrum was obtained under conditions of proton noise decoupling. Dioxane was omitted in this spectrum to simplify the picture.

taken from these solutions or from dilutions of them to meet the demands of the assays. These samples were not dried to constant weight.

The ¹³C NMR spectrum (Fig. 2) of HI-B showed the presence of 10 different carbon atoms in the polymer. Five of these carbon resonances showed resolvable ¹³C-³¹P coupling. Only one resonance (107.5 ppm) occurred in the range of chemical shifts typical of anomeric carbon atoms. A peak near 83 ppm was assigned to C-4 of the ribosyl moiety by comparison to the published spectra of the methyl ribofuranosides (26). The remaining assignments (see Table II) are consistent with the spectra of these models as well as that of ribitol (27). Finally, the assigned ¹³C-³¹P coupling constants are consistent with results obtained from nucleotide studies (28, 29). The assignments presented in Table II should be considered tentative since an extended study of model compounds has not been done.

The intact polymer consumed 2 mol of periodate per mol of repeating unit consisting of ribose, ribitol, phosphate, and sodium (Fig. 3). Tests for the production of formaldehyde were negative; however, 1 mol of formic acid was found per mol of the repeating unit.

The products of acid and base hydrolysis were subjected to analysis by paper and ion exchange chromatography. Table III

TABLE II
Carbon 13 chemical shifts and ^{13}C - ^{31}P couplings of HI-B

Carbon	Ribose		Ribitol	
	δ_{TM}^a	J ^{13}C - ^{31}P	δ_{TM}^a	J ^{13}C - ^{31}P
1	107.5		67.5	6.1
2	(75.1)	4.9	71.7	7.9
3	(74.6)	3.1	(70.9)	
4	82.8	6.7	(72.3)	
5	68.2		(69.5)	

^a In parts per million from tetramethylsilane (TMS), calculated from 1,4-dioxane as internal standard; $\delta_{\text{TM}} = \delta_{\text{dioxane}} + 67.4$. Resonances in parentheses cannot be assigned specifically.

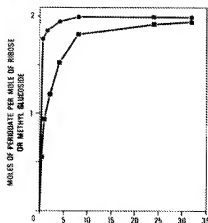


FIG. 3. Periodate oxidation of methyl- α -D-glucoside (■) and HI-B (●). The reaction mixture containing 1.5 μ mol of sodium phosphate buffer, pH 6.5, approximately 0.5 mmol of methyl- α -D-glucoside or ribose (in HI-B), and 1.0 mmol of sodium periodate for methyl- α -D-glucoside or 1.5 mmol of sodium periodate for HI-B in a total volume of 100 ml was incubated in the dark for the times indicated. At each time, a 5-ml aliquot was removed, 10 ml of saturated sodium bicarbonate, 2 ml of 20% potassium iodide, and 20 ml of 0.01 M sodium arsenite solution were added and the mixture back-titrated with 0.01 M iodine solution.

gives the R_{ribitol} mobility values on paper of substances giving reactions with aniline-oxalate reagent, which is very sensitive for reducing pentoses, and with periodate-Schiff reagent, which is very sensitive for sugar alcohols. When vigorously hydrolyzed with acid (17 or 66 hours at 100°), HI-B had one product which had the same mobility as 1,4-anhydribose. Occasionally, a trace of ribitol was present. Under these vigorous hydrolysis conditions, ribose was destroyed and ribitol was converted to 1,4-anhydribose (Fig. 4). Using mild acid hydrolysis (1.0 M HCl at 100° for 20 min or 0.1 M HCl at 100° for 10 min), several phosphate-containing compounds were detected. One indicated by its intense yellow color with periodate-Schiff reagent and reaction with aniline-oxalate reagent that it was ribose 3-phosphate. In reactions with periodate-Schiff reagent, standard solutions of ribose 5-phosphate gave a purple color, and ribose 3-phosphate an intense yellow color. No reaction was observed between ribose 2-phosphate and the periodate-Schiff reagent in the 5-min oxidation period used on chromatograms. Ribose 2- and 3-phosphates were also identified in acid hydrolysates with ion exchange chromatography on Dowex 1-Cl by comparison of elution profiles with standard ribose 2- and 3-phosphates. Ribose, ribitol, and 1,4-anhydribose were also found in the hydrolysates (Table IV).

TABLE III
 R_{mob} values

Sample	Solvent system		
	C	D	A
Ribitol.....	1.00	1.00	1.00
D-Ribose.....	1.11	1.16	
1,4-Anhydribose.....	1.45	1.68	1.11
HI-B ^a	0.98, 1.44	1.04, 1.64	1.12

^a Descending paper chromatography of 66-hour acid hydrolysates of HI-B.

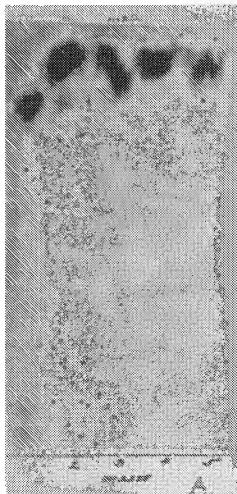


FIG. 4. Descending paper chromatography was done in Solvent A (n-propyl alcohol/ammonia ($d = 0.88$)/water, 6/3/1) for 24 hours. Components were detected with periodate-Schiff reagent. Samples are identified as follows: 1, unhydrolyzed ribitol; 2, hydrolyzed ribitol; 3, hydrolyzed ribitol 1 (5)-phosphate; 4, hydrolyzed HI-B; 5, anhydribose. Samples 2, 3, and 4 were hydrolyzed with 2 N HCl for 17 hours at 100° .

When basic hydrolysates were chromatographed, five products were observed. All had mobilities slower than ribitol and ribose in all solvent systems (Table V). Two of the products (B-2, B-5, Table V) had no reducing capacity, but reacted strongly with periodate-Schiff reagent, which indicated the presence of vicinal hydroxyl groups. Only one of these (B-2) had phosphate present. A third product (B-1) contained phosphate and had a mobility in Solvent A similar to those reported for bisphosphates (30).

TABLE IV
Components of acid and basic hydrolysates of HI-B

Compound ^a	Method of hydrolyses			
	0.1 N HCl, 10 min	1.0 N HCl, 20 min	0.5 N NaOH, 4 hrs, 25°	2.0 M HCl, 17 or 66 hrs
Ribose	+	+	—	—
Ribitol	+	+	—	Trace
Ribose-3-PO ₄	+	+	Trace	—
Ribose-2-PO ₄	+	+	—	—
1,4-Anhydribose	+	+	—	+
Ribosyl-ribitol ^b	—	—	+	—
Ribosyl-ribitol-P ^b	—	—	+	—

^a Compounds were identified by descending paper chromatography and ion exchange chromatography on Dowex 1-Cl of hydrolysates of HI-B. All acid hydrolysates were hydrolyzed at 100°.

^b Compounds tentatively identified.

TABLE V
R-values of basic hydrolysates of HI-B

Component ^a	Solvent A	Solvent B	Solvent C	Concentration
B-1	0.17	0	0.14	Minor
B-2	0.48	0.13	0.40	Major
B-3	ND ^b	0.19	0.54	Trace
B-4	ND	0.51	ND	Trace
B-5	0.94	0.86	0.71	Major

^a Components numbered in order of migration from the origin.

^b ND, not detected.

This product exhibited no reducing capacity with aniline-oxalate reagent, but had a strong positive reaction with periodate-Schiff reagent. The fourth component (B-3) stained yellow with periodate-Schiff and was identified as ribose 3-phosphate. The fifth component (B-4) was not identified.

DISCUSSION

Our results confirmed the presence of ribose and phosphate in equimolar quantities. In addition, the presence of ribitol was indicated by its ease of conversion on acid hydrolysis to 1,4-anhydribose. The ribose standard was destroyed under vigorous acidic conditions and not converted to any product that reacted with aniline-oxalate or periodate-Schiff reagents. Hydrolysis of HI-B under the same conditions yielded only 1,4-anhydribose and a trace of ribitol. The 1,4-anhydribose was distinguished easily from glycerol by the periodate-Schiff reagent. Glycerol gave a purple color within 5 min, while 1,4-anhydribose gave an intense blue color after 30 min (31). Ribitol was distinguished from other pentitols and hexitols by its ease of conversion to 1,4-anhydribose under mild acid conditions, such as 1.0 M HCl at 100° for 20 min (13, 32). Quantitative determinations of ribitol showed that it was present in equal molar amounts when compared to ribose or phosphate regardless of the molecular size of the preparation (Table I). These determinations were done only on highly purified samples of HI-B, and assays were corrected for ribose.

Under mild acid conditions, ribose and ribitol were identified by paper chromatography. In addition, a hydrolysis product was identified as ribose 3-phosphate. Although ribose 2-phosphate was also present in the acid hydrolysate, this could have arisen in

a manner analogous to the acid hydrolysis of RNA. Enzymatic work done by Rosenberg and Zamenhof (4) found HI-B to be hydrolyzed by RNase 2500 times slower than RNA. From these data they postulated that the 2 hydroxyl of ribose was spatially oriented similar to the pyrimidine nucleotides and would place the phosphate in position 3 on ribose.

Since ribitol was found in acid hydrolysates, the fact that a sugar phosphate was present suggested a structure like the type 2 ribitol teichoic acids, which have a repeating unit of glucosylaldehyde phosphate in the main chain as reported by Archibald (31). Chemical evidence for the presence of ribosylribitolphosphate was based on information from the analysis of mild alkaline hydrolysates. Two major components separated by paper chromatography were nonreducing but reacted with periodate-Schiff reagent. One (B-2) contained phosphate as indicated by its reaction with molybdate reagent. The other (B-5) had a mobility slightly less than ribitol and did not contain phosphate. A non-ionic substance was eluted with water from a Dowex 1 column which had the same mobility as component B-5. Quantitative analysis showed ribose and ribitol to be present in equimolar quantities. This substance was assigned the structure of ribosyl-ribitol and is analogous to the glucosylribitol reported for ribitol teichoic acids by Armstrong *et al.* (30). A component was eluted from the Dowex 1 column with 0.06 M NH₄Cl containing 0.004 M K₃B₄O₇ and found to contain equimolar quantities of ribose, ribitol, and phosphate. It had the same mobility as B-2 which was faster than the phosphate-containing standards (ribose 2-, 3-, or 5-phosphates). Periodate oxidation studies of the HI-B preparations showed that no formaldehyde was formed but that 1 mol of formic acid was produced when the 2 mol of periodate were consumed. Such results require the presence of 3 adjacent carbon atoms with unsubstituted hydroxyl groups. Earlier workers (1, 4) reported that oxidizable impurities impaired the quantitative determination of periodate uptake. Therefore, these workers determined the amount of ribose before and after periodate treatment and found no decrease in the amount of ribose. Periodate oxidation of our proposed structure would not destroy the ribose, since the vicinal hydroxyl groups are present only in the ribitol moiety. Also, unless the ribosyl linkage were hydrolyzed, our proposed structure would be nonreducing, as was the polyribosephosphate (PRP) structure.

The fact that the ¹³C NMR spectrum clearly showed 10 non-equivalent carbon atoms effectively rules out the symmetrical polyribosephosphate structure proposed earlier (1, 5). In addition, the polyribosephosphate structure was linked through the 3- and 5-hydroxyls, a situation which would place C-4 vicinal to two phosphates. In such a circumstance, this carbon would be coupled to two phosphorus nuclei; no such resonance occurs in the ¹³C NMR spectrum. The suggestion that HI-B is comprised of a mixture of polyribosephosphate and polyribitolphosphate (9) is also unlikely, since according to our data, ribose, ribitol, and phosphate are present in equimolar quantities and such a mixture would require two molar equivalents of phosphate per mol of ribose or ribitol. Also, at least 8 of the 10 carbon atoms shown in the ¹³C NMR spectra would show ¹³C-P coupling in this situation. In contrast, the ¹³C data were wholly consistent with the structure proposed here (Fig. 5). Five carbon atoms (2, 3, and 4 of the ribose portion; 1 and 2 of the ribitol) showed carbon-phosphorus coupling. The anomeric carbon resonance showed no such coupling indicating that the phosphate was not linked to carbon 2 of the ribosyl unit.

Baddley (3) stated that teichoic acids, in which ribitolphosphate repeating units were joined together by phosphodiester

